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Abstract: Yersinia enterocolitica biotype 1A strains are frequently isolated from the environment, foods, and animals, and also from humans with yersiniosis. There are controversial reports on the pathogenicity of biotype 1A strains. In this study, 811 fecal samples from asymptomatic humans from Switzerland were studied for the presence of Y. enterocolitica. Nine (1.1%) of the 811 samples were positive for Y. enterocolitica 1A. These strains were compared with 12 Y. enterocolitica 1A strains from Swiss patients with diarrhea isolated in the same year. Almost all (20/21) Y. enterocolitica 1A strains carried the ystB gene, seven strains carried the hreP gene, and none carried the ail, ystA, myfA, yadA, or virF genes. Most (17/21) Y. enterocolitica 1A strains belonged to two major clusters, A and B, by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Strains of cluster B were only isolated from humans with diarrhea; however, ystB and hreP genes were detected in strains from both clinical and non-clinical samples and from strains of clusters A and B. Using ribotyping, six restriction patterns among biotype 1A strains were obtained with HindIII enzyme. The most common ribotype (RT I) was found in strains isolated from humans with and without diarrhea. All biotype 1A strains had a unique NotI profile by pulsed-field gel electrophoresis (PFGE), showing a very high genetic diversity. In this study, Y. enterocolitica 1A strains from clinical and non-clinical samples could not be clearly differentiated from each other. More research is needed in order to prove that biotype 1A strains are a primary cause for human yersiniosis and not only a secondary finding.

DOI: https://doi.org/10.1007/s10096-013-1820-1

Characteristics of *Yersinia enterocolitica* biotype 1A strains isolated from patients and asymptomatic carriers

R. Stephan[1], S. Joutsen[2], E. Hofer[1], E. Säde[2], J. Björkroth[2], D. Ziegler[3] and M. Fredriksson-Ahomaa[2]

[1]Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, Zurich, Switzerland

[2]Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki, Finland

[3]Mabritec AG, Riehen, Switzerland

Running title: *Yersinia enterocolitica* biotype 1A in humans

Corresponding author:

M. Fredriksson-Ahomaa, Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki, Finland, maria.fredriksson-ahomaa@helsinki.fi

Abstract

Yersinia enterocolitica biotype 1A strains are frequently isolated from the environment, foods and animals but also from humans with yersiniosis. There are controversial reports on the pathogenicity of biotype 1A strains. In this study, 811 fecal samples from asymptomatic humans from Switzerland were studied for the presence of *Y. enterocolitica*. Nine (1.1%) of the 811 samples were positive for *Y. enterocolitica* 1A. These strains were compared with 12 *Y. enterocolitica* 1A strains from Swiss patients with diarrhea isolated in the same year. Almost all (20/21) *Y. enterocolitica* 1A strains carried the *ystB* gene, 7 strains carried the *hreP* and none carried the *ail*, *yst*A, *myf*A, *yad*A or *vir*F gene. Most (17/21) *Y. enterocolitica* 1A strains belonged to two major clusters, A and B, by MALDI-TOF. Strains of cluster B were only isolated from humans with diarrhea, however, *yst*B and *hre*P genes were detected in strains from both clinical and non-clinical samples and from strains of cluster A and B. Using ribotyping, 6 restriction patterns among biotype 1A strains were obtained with *Hind*III enzyme. The most common ribotype (RT I) was found in strains isolated from humans with and without diarrhea. All biotype 1A strains had a unique *Not*I profile by PFGE showing a very high genetic diversity. In this study, *Y. enterocolitica* 1A strains from clinical and non-clinical samples could not clearly be differentiated from each other. More research is needed to prove that biotype 1A strains are a primary cause for human yersiniosis and not only a secondary finding.

Key words: *Yersinia* spp.; fecal samples; identification; pathogenicity

Introduction

Yersinia enterocolitica is an important enteric bacterium causing gastrointestinal problems, long-term sequelae like reactive arthritis, and sometimes septicemia due to blood-transfusion [4, 9]. *Y. enterocolitica* represents 6 biotypes (1A, 1B, 2-5) [2]. Strains belonging to biotypes 1B and 2 to 5 carry the virulence plasmid (pYV) and the chromosomal genes *ail*, *ystA*, *myfA* and *hreP*, and are thus considered pathogenic to humans and animals. Strains belonging to biotype 1A are considered nonpathogenic because they do not carry the pYV and the important chromosomal virulence genes are missing [3, 5].

Biotype 1A strains are widely distributed in the environment and have frequently been isolated from samples of food and animal origin [2]. However, strains of biotype 1A have also been isolated from symptomatic humans [6, 18, 20]. In Finland and Switzerland, biotype 1A strains are common findings in feces of diarrheic humans [6, 20]. Still septicemia and reactive arthritis have only rarely been reported [2].

Accurate identification of *Y. enterocolitica* can be difficult if only biochemical tests are used [20]. Especially, *Y. massiliensis, Y. mollaretii, Y. bercovieri* and *Y. rohdei* are very easily misdiagnosed as *Y. enterocolitica* with phenotypic methods. Information of the biotype and virulence genes is needed for proper assessment of potential pathogenicity of the *Yersinia* strain [20]. *Y. enterocolitica* 1A strains are serologically very heterogeneous [2] and they show clearly wider genetic diversity than the human pathogenic strains belonging to biotypes 2 and 4 [6, 15]. *Y. enterocolitica* 1A strains have been reported sometimes to carry chromosomal virulence genes like *myfA, ystB* and *hreP* [3].

There are controversial reports on the pathogenicity of clinical and non-clinical *Y. enterocolitica* biotype 1A strains and thus, *Yersinia* strains from humans with and without diarrhea were collected in Switzerland during 2011 for further characterization using phenotypic and genotypic methods.

Materials

In total, 811 fecal samples from asymptomatic humans collected in 2011 in Switzerland were studied for the presence of *Yersinia* spp.. The samples were from humans between the age of 20 and 60. Most (641/811) of the samples were from males, 166 samples were from females and for 4 samples the gender was not known. Furthermore, 26 *Yersinia* spp. strains isolated in 2011 from patients with diarrhea in Switzerland were characterized and compared with the strains isolated from asymptomatic humans of the same year (Table 1).

Methods

About a 1-g fecal sample was mixed in 9 ml PMB (peptone broth supplemented with 1% mannitol and 0.15% bile salts) [16]. Cold enrichment at 4°C for 3 weeks was used for all samples before plating on *Yersinia*-selective CIN (cefsulodin-irgasan-novobiocin) agar (Oxoid AG, Basel Switzerland). The CIN plates were incubated at 30°C for 24 to 48 h. Presumptive positive colonies were subcultured on blood agar and then tested for the urease enzyme. Urease-positive colonies were identified with API 20E and MALDI-TOF [6, 22].

Y. enterocolitica isolates were bio- and serotyped [6]. The biotype was determined using pyrazinamidase and tween activity, esculin hydrolysis, indole production, and salicin, xylose

and trehalose fermentation and serotyping was carried out with slide agglutination using commercial *Y. enterocolitica* O:1-O:3, O:5, O:9 (Denka Seiken, Tokyo, Japan) and O:27 antisera (Sifin, Berlin, Germany).

Seven genes were studied by PCR: two virulence genes (*yad*A and *vir*F) located on the virulence plasmid (pYV) of the pathogenic *Yersinia* spp. and 5 chromosomal virulence genes (*ail, yst*A*, yst*B*, myf*A and *hre*P) [3, 12, 23, 24]. The DNA was released from bacterial colonies by heating at 99°C for 10 min, and 1 µl of this liquid was added to 19 µl of the master mix, which contained 1x ready -to-use mix (iQ™ SYBR Green Supermix, Bio-Rad, Hercules, CA) and 200 nM of primers. All genes were studied separately in a single PCR. The fluorescence intensity of the SYBR Green and the melting curve analysis were studied using the CFX96 system (Bio-Rad). A threshold cycle (Ct) under 30 and a specific melting temperature (Tm) indicated a positive result.

Moreover, the *Yersinia* strains (one isolate per sample) were genotyped. The 16S and 23S restriction fragment length polymorphism (ribotyping) of the strains was studied using *Hind*III restriction enzymes [7] and *Not*I enzyme was used for pulsed-field gel electrophoresis [8]. Dice correlation coefficient and UPGMA clustering were used for constructing the dendrogram (Fig. 1).

Results

Nine (1.1%) of the 811 fecal samples from asymptomatic humans were *Y. enterocolitica* biotype 1A positive. *Y. enterocolitica* was the only *Yersinia* species isolated from the samples and biotype 1A the only biotype identified.

120

121 In total, 26 clinical *Y. enterocolitica* strains were sent to the *Yersinia* reference laboratory in
122 Switzerland during the year 2011. Fourteen (54%) of the 26 strains belonged to biotypes 2 or
123 4 and 12 (46%) to biotype 1A (Table 1). Seven (50%) of the 14 strains of biotype 2 or 4 but
124 only one (8%) of the 12 biotype 1A strains were isolated from humans under 20 year old.
125
126 Biotype 1A strains have frequently been isolated in fecal samples of humans with diarrhea in
127 Switzerland (Table 2). In 2011, biotype 1A was the most common type (46%) found in the
128 fecal samples of humans with diarrhea followed by biotypes 4 (35%) and 2 (19%).
129
130 Most (86%) of the 14 *Y. enterocolitica* strains belonging to biotype 2 or 4 were identified
131 with a high ID% using API 20E (Table 3). Only two of these strains (14%) had a low or no
132 ID% (https://apiweb.biomerieux.com/servlet/Authenticate). Five (24%) of the 21 *Y.*
133 *enterocolitica* biotype 1A strains could not be identified with a high ID% using API 20E. One
134 of these strains was even identified as *Serratia marcescens*. Using MALDI-TOF, all *Y.*
135 *enterocolitica* biotype 1A strains were identified as *Y. enterocolitica* and were distinguished
136 from *Y. enterocolitica* biotype 2 and 4 strains.
137
138 All 14 *Y. enterocolitica* strains of biotype 2 or 4 carried the *ail, ystA, myfA* and *hreP* genes in
139 the chromosome but were *ystB* negative (Table 4). Most (79%) of the biotype 2 and 4 strains
140 carried also *yadA* and *virF* genes on the pYV. Almost all (95%) *Y. enterocolitica* 1A strains
141 carried *ystB* in the chromosome, only one strain was negative. The ribotype pattern (RT VI)
142 of the *ystB*-negative strain differed clearly from the ribotype patterns (RT I-V) of the *ystB*-
143 positive strains (Fig. 1). The *hreP* was detected in 7 (33%) *Y. enterocolitica* 1A strains (Table
144 5). None of the *Y. enterocolitica* 1A strains carried the *yadA* or *virF* gene.

Most (81%) *Y. enterocolitica* 1A strains belonged to two major clusters, A and B, by MALDI-TOF. All but one strain from humans without diarrhea belonged to cluster A and strains of cluster B were only isolated from clinical stool samples (Table 5). Strains carrying *ystB* and *hreP* were found in clinical and non-clinical strains and in strains from both A and B clusters. Using ribotyping, 6 different restriction patterns (ribotypes), RT I-VI, were obtained with *Hind*III enzyme (Fig. 1). Ten (48%) of the *Y. enterocolitica* 1A strains expressed ribotype I and they were isolated from humans with and without diarrhea (Table 5). Ribotype II was found in 3 strains which were all from clinical samples. Different *Not*I profiles were obtained in all biotype 1A strains and no clear clustering between the strains according source or any other identifiable determinant could be seen.

Discussion

The prevalence of *Y. enterocolitica* 1A in fecal samples from asymptomatic humans was only 1.1%, which is surprisingly low since *Y. enterocolitica* 1A and other non-pathogenic *Yersinia* spp. are frequently isolated from different food samples [2].

During 2011, 26 *Y. enterocolitica* strains from humans with diarrhea were sent to the national reference laboratory for *Yersinia*. Three biotypes (1A, 2 and 4) were identified. The most common biotype was 1A which has frequently been isolated from clinical fecal samples of symptomatic patients in Switzerland during the last decade [6]. Direct culturing is mostly used for clinical samples in Switzerland which indicates that the number of *Y. enterocolitica* 1A is higher in fecal samples from humans with diarrhea compared to them without diarrhea. Biotype 1A strains have also been reported to be common in Finnish patients. However, in

170 Finland, cold enrichment, which supports the growth of all psychrotrophic *Yersinia* spp., is
171 used also for clinical samples [20].
172
173 *Y. enterocolitica* strains of biotype 2 or 4 were only isolated from fecal samples of humans
174 with diarrhea which shows that asymptomatic humans do not usually shed strains of these
175 biotypes. Strains of biotypes 2 and 4 were frequently isolated from young patients (under 20
176 years). *Y. enterocolitica* 1A strains were isolated from fecal samples from both symptomatic
177 and asymptomatic humans. In Finland, the symptoms and sources of patients with *Y.*
178 *enterocolitica* 1A strains differed from those patients with strains of biotypes 2 and 4. The
179 patients with biotype 1A strains were adults with more long-lasting, unspecific symptoms
180 which suggest that the original cause of illness may have been other than *Y. enterocolitica* 1A
181 [13].
182
183 Identification of *Y. enterocolitica* strains by phenotypic method has shown to be very
184 laborious and the accurate identification difficult [20]. However, the authors reported that by
185 combining API 20E and biotyping, *Y. enterocolitica* strains belonging to biotypes 2-5 can be
186 identified reliably. In this study, *Yersinia* strains were identified with API 20E and MALDI-
187 TOF. Some discrepancies in identification of *Y. enterocolitica* occurred if only API 20E was
188 used. All *Y. enterocolitica* 1A strains were differentiated from *Y. enterocolitica* biotype 2 and
189 4 strains by MALDI-TOF, which is a convenient method to identify a high number of
190 bacterial strains rapidly [22].
191
192 Distribution of the virulence genes differed between the biotype 1A strains and biotype 2 and
193 4 strains. All *Y. enterocolitica* strains of biotype 2 or 4 carried the chromosomal *ail, ystA,*
194 *myfA* and *hreP* genes. Furthermore, *virF* and *yadA* located on the pYV were detected in most

195 of biotype 2 and 4 strains. All *Y. enterocolitica* 1A strains were *virF, yadA, ail*, *ystA* and *myfA*
196 negative. The pYV has so far not been found in *Y. enterocolitica* 1A strains and thus, *virF*
197 and *yadA* have also not been detected in biotype 1A strains. In our earlier study, one of the 51
198 human clinical *Y. enterocolitica* 1A strains carried the *ail* in Switzerland (Fredriksson-
199 Ahomaa et al., 2012). Recently, the *ail* was also detected in some biotype 1A strains in
200 Germany and Finland [14, 19]. However, *ail* and *ystA* have very seldom been detected among
201 biotype 1A strains [3]. All but one *Y. enterocolitica* 1A strain carried the *ystB*. It has been
202 demonstrated that *Y. enterocolitica* can produce heat-stable enterotoxins. YstB is usually
203 produced by strains belonging to biotype 1A and the enterotoxin YstA by strains belonging to
204 biotypes 1B and 2-5. Singh and Virdi [21] showed that *ystB* gene is widely distributed among
205 human clinical isolates and that production of YstB enterotoxin can be induced at the
206 conditions found in ileum (37°C, pH 7.5) indicating that YstB is an important virulence
207 determinant in biotype 1A strains [21]. However, in this study, *ystB* was also detected in all
208 *Y. enterocolitica* 1A strains isolated from asymptomatic humans. Furthermore, *hreP* gene was
209 detected among clinical and non-clinical strains. The strain carrying the *yst*B and *hre*P genes
210 may have some pathogenic potential but more research is needed. The *myfA* has shown to be
211 more predominant in Indian biotype 1A strains than in European strains. Bazilla et al. [1]
212 sequenced two *Y. enterocolitica* 1A strains and found in both strains *ystB, myfA* and *hreP* but
213 not *ail* and *ystA* genes. However, *myfA* in the both biotype 1A strains showed sequence
214 variability and differed from highly conserved *myfA* in biotype 1B and 4 strains [1]. This
215 sequence variability may explain the failure to detect the *myfA* in biotype 1A strains.
216
217 Two major clonal groups of *Y. enterocolitica* 1A have been reported earlier by different
218 genotypic and phenotypic methods [10, 11, 17, 22]. Furthermore, Bhagat and Virdi have
219 shown a correlation between the distribution of virulence-associated genes *myfA, ystB* and

220 *hreP* and the clonal groups [3]. In this study, *Y. enterocolitica* 1A strains were grouped into
221 two major clusters using MALDI-TOF; most of the non-clinical strains were grouped into
222 cluster A and most of the clinical strains into cluster B. However, no correlation between the
223 distribution of the virulence-associated genes and the clonal groups were seen; *ystB* and *hreP*
224 were distributed in strains of both clonal groups. In the earlier study by Bhagat and Virdi [3],
225 the distribution of virulence-associated genes between clinical and non-clinical strains did not
226 significantly differ, which is in accordance with our results. Furthermore, no clear clustering
227 of non-clinical and clinical strains was obtained by ribotyping and PFGE. All 1A strains
228 revealed different *Not*I profiles by PFGE showing a high genetic diversity among these
229 strains. These results could not show any clear difference between *Y. enterocolitica* 1A
230 strains isolated from humans with diarrhea and without diarrhea. More research is needed to
231 prove the significance of biotype 1A strains in human yersiniosis.

232

233

234 **Table 1** Age and gender distribution of humans with and without diarrhea of which *Y.*

235 *enterocolitica* were isolated in 2011 in Switzerland

236

Diarrhea	Biotype	No. of strains	Gender		Age (years)		
			Female	Male	< 20	20-50	>50
Yes	2 or 4	14	5	9	7	5	2
Yes	1A	12	5	7	1	7	4
No	1A	9	1	8	0	8	1

237

238 **Table 2** Biotypes of 141 *Y. enterocolitica* strains isolated from fecal samples of patients with

239 diarrhea during 2003 and 2011 in Switzerland

240

Year	Number of strains (%)									
	1A		2		3		4		NT	
2003[a]	5	(38)	4	(31)	0		4	(31)	0	
2004[a]	4	(44)	2	(22)	0		3	(33)	0	
2005[a]	4	(21)	4	(21)	1	(5)	8	(42)	2	(11)
2006[a]	9	(45)	2	(10)	0		9	(45)	0	
2007[a]	10	(71)	1	(7)	0		3	(21)	0	
2008[a]	4	(44)	1	(11)	0		4	(44)	0	
2009[a]	5	(45)	3	(27)	0		3	(27)	0	
2010[a]	8	(40)	5	(25)	1	(5)	6	(30)	0	
2011	12	(46)	5	(19)	0		9	(35)	0	

241

242 NT, not typable

243 [a]Extra-intestinal results have been excluded from the data published by Fredriksson-Ahomaa

244 et al., 2012

245 **Table 3** Identification of *Y. enterocolitica* (YE) isolated from fecal samples of humans with
246 and without diarrhea in 2011 in Switzerland
247

Diarrhea	No. of strains	API 20E		Biotype (BT)	MALDI-TOF
		Code	*Yersinia enterocolitica* ID %		
Yes	5	1015523	93.8	BT 4	YE BT 2-4
	2	1015522	89.4		
	1	0115523	99.9		
	1	3055723	*Yersinia enterocolitica*[a]		
Yes	3	1155723	98.3	BT 2	YE BT 2-4
	1	1155323	98.9		
	1	1355723	46.8		
Yes	7	1155723	98.3	BT 1A	YE BT 1A
	1	1155763	96.9		
	2	1355723	46.8		
	1	5757723	*Serratia marcescens*[a]		
	1	3355723	*Yersinia enterocolitica*[a]		
No	8	1155723	98.3		
	1	1355723	46.3		

248

249 [a]The significant species without ID%

250 **Table 4** Distribution of virulence-associated genes among *Y. enterocolitica* strains isolated
251 from fecal samples of humans with and without diarrhea in 2011 in Switzerland
252

Diarrhea	No. of strains	Bio-type	Serotype	Virulence-associated genes						
				yadA	*virF*	*ail*	*ystA*	*ystB*	*myfA*	*hreP*
Yes	9	4	O:3 (9)	7	7	9	9	0	9	9
	1	2	O:5,27 (1)	1	1	1	1	0	1	1
	4	2	O:9 (4)	3	3	4	4	0	4	4
	7	1A	O:5 (3), O:8 (2), O:5,8 (1), NT (1)	0	0	0	0	7	0	0
	4	1A	O:8 (1), O:9 (1), O:5,8 (1), NT (1)	0	0	0	0	4	0	4
	1	1A	O:8 (1)	0	0	0	0	0	0	0
No	6	1A	O:5 (3), O:8 (2), NT (1)	0	0	0	0	6	0	0
	3	1A	O:5 (1), O:8 (1), NT (1)	0	0	0	0	3	0	3

253

254 **Table 5** Distribution of different MALDI-TOF clusters, ribotypes and virulence genes among
255 the *Y. enterocolitica* 1A strains isolated from fecal samples of humans with and without
256 diarrhea in 2011 in Switzerland
257

Diarrhea	No. of strains	MALDI-TOF	Ribotype (*Hind*III)	No. of *ystB*-positive strains	No. of *hreP*-positive strains
No	4	A	I	4	1
	2	A	IV	2	1
	1	A	NT[a]	1	0
	1	A	III	1	0
	1	E	I	1	1
Yes	4	B	I	4	2
	2	B	II	2	2
	1	B	III	1	0
	1	A/B	II	1	0
	1	A/B	IV	1	0
	1	C	V	1	0
	1	D	I	1	0
	1	F	VI	0	0

258
259 [a]This strain was restricted with *Eco*RI and it showed an identical ribotype with the *Y.*
260 *enterocolitica* 1A reference strain CCUG 52868
261

262

Fig. 1 The ribotypes (RT) I-VI found among *Y. enterocolitica* 1A strains with *Hind*III restriction enzyme

Conflict of interest:

The authors declare that they have no conflict of interest.

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